40 94 *

Applied
Microbiology

Effect of Some Inhibitors Derived from Nitrite on Macromolecular Synthesis in *Bacillus cereus*

J. N. HANSEN* AND R. A. LEVIN

Department of Chemistry, University of Maryland, College Park, Maryland 20742

The effect of several inhibitors derived from nitrite on incorporation of [14C]uracil into ribonucleic acid of *Bacillus cereus* during outgrowth and vegetative growth has been determined. A heat-induced inhibitor presumably of the Perigo type was compared with the nitrosothiols of thioglycolate and β-mercaptoethanol. All were found to effectively inhibit uracil incorporation at all stages tested. Phase-contrast microscopy revealed that inhibition of morphological events occurred either before germination or during early outgrowth, depending on inhibitor concentration. It was also found that a precursor to the heat-induced inhibitor could be isolated from dialyzable tryptone (a pancreatic digest of casein), which chromatographed as a single species on diethylaminoethyl-cellulose and Sephadex G-25, with an apparent molecular weight of about 2,000.

Nitrite is commonly used as an antimicrobial agent, but little is known about its molecular mode of action. This is partly due to the wide variety of reactions in which nitrite and nitrous acid can participate. Although the conditions for optimal effectiveness of nitrite have been studied (5, 6, 17, 20), the chemical reactivity of the inhibiting species is not known. The earlier observation that media that contain nitrite become dramatically more inhibitory toward bacterial growth when heated implies that one or more new chemical species have been produced (15). It has been suggested that these substances, called Perigo inhibitors, are responsible for the antimicrobial properties of nitrite-curing salts used as preservatives. Evidence has been presented which indicates that the heated media may contain substances such as Roussin black salt (1) and nitrosothiols (9, 12, 13). However, it is possible that heat-induced Perigo inhibitors are distinct from these compounds.

The potential value of these inhibitors as preservatives makes them attractive for mechanistic studies. We have therefore examined and compared the effects of a heat-induced inhibitor, presumably of the Perigo type, with those of nitrosothiols on germination, outgrowth, and macromolecular synthesis in *Bacillus cereus*. The excellent synchrony of germination and outgrowth in this organism provides a convenient means for evaluating inhibitor effects on bacterial spores. We have also fractionated tryptone to obtain a purified precursor to the heat-induced inhibitor.

MATERIALS AND METHODS

Reagents. Sephadex G-25, blue dextran, insulin, ribonuclease S peptide, bacitracin, and vasopresin were obtained from Sigma. Tritium oxide was obtained from New England Nuclear Corp. Proteinase K was from EM Laboratories.

Organism and preparation of spores. The organism employed for the work was *B. cereus* T. Cells were grown and sporulated in G medium (7) supplemented with calcium (26). They were washed (26), mented with calcium (26). They were washed (26), lyophilized, and stored at −20 C. For outgrowth studies, the spores were weighed out and suspended in distilled water with a glass homogenizer. They were heat shocked for 2 h in a 65 C water bath and centrifuged. The spores were then suspended in water, and an aliquot was added to the outgrowth medium to initiate germination. The final concentration of spores was 0.12 mg/ml, and growth experiments were carried out in a 30 C shaking water bath at 75 rpm unless indicated otherwise.

Preparation of dialyzable tryptone. A 200-g amount of tryptone (a pancreatic digest of casein obtained from Difco) was added to 500 ml of water. The mixture was heated with stirring to about 90 C and allowed to stand for at least 10 h at 4 C. The suspension was centrifuged and the pellet was discarded. The supernatant was put into 8-cm-wide dialysis tubing and dialyzed for 48 h with stirring against 500 ml of water at 4 C. The outer material was recovered, and the dialysis was repeated against a change of water. The outer material was combined with that from the first dialysis. This dialyzable tryptone was stored in frozen aliquots.

Outgrowth media. Dialyzable tryptone was diluted as indicated for each experiment. Fully supplemented CDGS medium was based on that of Nakata (14), as modified by Rana and Halvorson (18) and Rodenberg et al. (22). It contained CDGS salts,

CDGS amino acids, supplementary amino acids and bases, 0.5 g of adenosine, 0.01 g of thymidine, 0.1 g of alanine, and 4 g of glucose per liter.

Preparation of heat-induced inhibitor. Sodium nitrite and sodium thioglycolate were added to 40 ml of dialyzable tryptone to give final concentrations of 27 and 8.8 mM, respectively. Heat-induced inhibitor (presumed to be of the Perigo type) was obtained by autoclaving this mixture at 110 C for 20 min. An identical mixture left unheated served as a control.

Preparation of nitrosothiols. Nitrosothiols of thioglycolate and β-mercaptoethanol were prepared from a solution containing 27 mM sodium nitrite and 8.8 mM of either sodium thioglycolate or β-mercaptoethanol. This mixture was acidified to a pH between 1 and 2 with concentrated HCl. After an hour, the solution containing the orange-red thionitrite (2, 4, 16, 23, 24) was titrated to neutrality with NaOH.

RESULTS

Phase-contrast microscopy of spores inhibited by heat-induced inhibitor. Heat-shocked spores were added to 20 ml of freshly prepared heat-induced inhibitor mixture. A second flask contained the unheated control mixture (see Materials and Methods). Samples were removed at intervals for observation by phase-contrast microscopy. Photomicrographs are shown in Fig. 1. In this rich medium the spores germinated rapidly and synchronously. Germination as monitored by phase contrast was complete within a few minutes in both the heated and control samples. After 0.5 h, the spores in the unheated control were swollen

and less phase-dark, a trend that continued through 1 h. At the end of 2 h, spores in the control flask had divided and were in the vegetative state, whereas spores in the sample flask had not changed appreciably after the initial germination stage. The inhibition remained unchanged through 18 h. After 24 h, however, an occasional elongated cell could be seen among the inhibited population.

Effect of increased concentrations of heat-induced inhibitor. Dialyzable tryptone was prepared as described above, except that the second dialysis was omitted. This resulted in a higher final concentration of dialyzable components. The corresponding inhibitor was formed using the same concentrations of nitrite and thioglycolate as before. A 10-ml amount was then added to a flask containing 10 ml of dialyzable tryptone, followed by heat-shocked spores. As monitored by phase-contrast microscopy, fewer then 1% of the spores germinated within 2 h to become phase-dark. After 16 h, the fraction germinated increased to about 2%. At the higher concentration, the sensitive step appears to be during germination, whereas lower concentrations inhibit after germination.

Effect of heat-induced inhibitor added before germination on [¹⁴C]uracil incorporation. Sample flasks contained 10 ml of supplemented CDGS and [¹⁴C]uracil (New England Nuclear Corp.) at an activity of 0.250 μCi/ml. Ten milliliters of heated inhibitor mixture was added to one set of flasks, and 10 ml of unheated
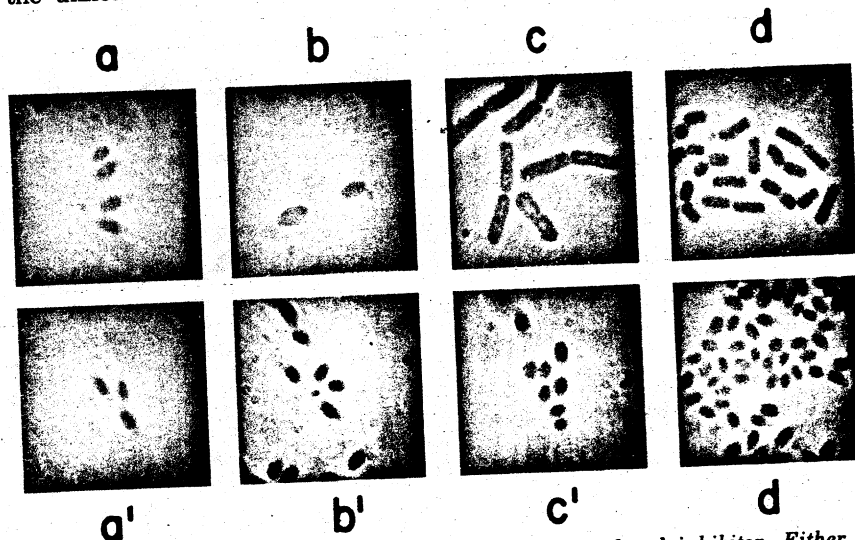

FIG. 1. *Phase-contrast microscopy of spores inhibited by heat-induced inhibitor. Either a heated or unheated mixture of dialyzable tryptone, nitrite, and thioglycolate was added to zero time to heat-shocked spores. (a) 0.5 h; (b) 1 h; (c) 2 h; (d) 18 h after the onset of germination. The heated samples are denoted by primes.*

mixture was added to the other set. Spores were added, and 1-ml samples were removed at intervals and quenched in 1 ml of cold 10% trichloroacetic acid containing 100 μg of unlabeled uracil per ml. The samples were filtered through nitrocellulose membrane filters (0.45-μm pore size), which were washed with 95% ethanol, dried, and counted. The scintillation fluid contained 4 g of 2,5-diphenyloxazole and 50 mg of p-bis-[2-(5-phenyloxazolyl)]benzene (New England Nuclear Corp.) per liter of toluene. The results are shown in Fig. 2. The sample containing the unheated control incorporated [^{14}C]uracil rapidly, whereas the sample containing the heat-induced inhibitor showed no incorporation.

Effect of heat-induced inhibitor added after germination. Sample flasks contained 12 ml of supplemented CDGS and [^{14}C]uracil. Spores were added to a concentration of 0.25 mg/ml. Samples of 0.5 ml each were removed during the first four time points. At 30 min after the onset of germination, 10 ml of either the heated or the unheated inhibitor mixture was added. Samples of 1 ml each were then removed. Radioactivity incorporation was determined as for Fig. 2. Figure 3 shows the pattern of [^{14}C]uracil incorporation which was obtained. Incorporation was halted in both cases; however, the control recovered rapidly and resumed incorporation, whereas the sample lost acid-precipitable counts. A control flask to which only dialyzable tryptone had been added

showed no lag in incorporation. Microscopic examination revealed that the sample to which the heat-induced inhibitor had been added did not develop further during the next 2 h, whereas the unheated control sample was vegetative within that time.

Effect of heat-induced inhibitor added during vegetative growth. Flasks containing 10 ml of supplemented CDGS medium were inoculated with 5 ml of early log-phase vegetative cells grown in the same medium. After 2.5 h, [^{14}C]uracil was added. Samples of 0.5 ml each were removed at the indicated times. At 37.5 min after the uracil addition, an equal volume of either the heated or unheated mixture was added, followed by removal of 1-ml volumes. The samples were all precipitated, filtered, and counted as for Fig. 2. Figure 4 shows the pattern of ^{14}C incorporation which was obtained. The sample to which unheated control had been added resumed incorporation after a short lag, whereas the sample to which the heated mixture had been added remained level. In contrast to the pattern observed in outgrowing spores, the acid-precipitable counts in the inhibited vegetative cells did not decrease during the subsequent incubation period. Continued observation during 36 h of incubation showed no recovery from inhibition, and some cell lysis occurred, whereas the unheated control sample grew normally.
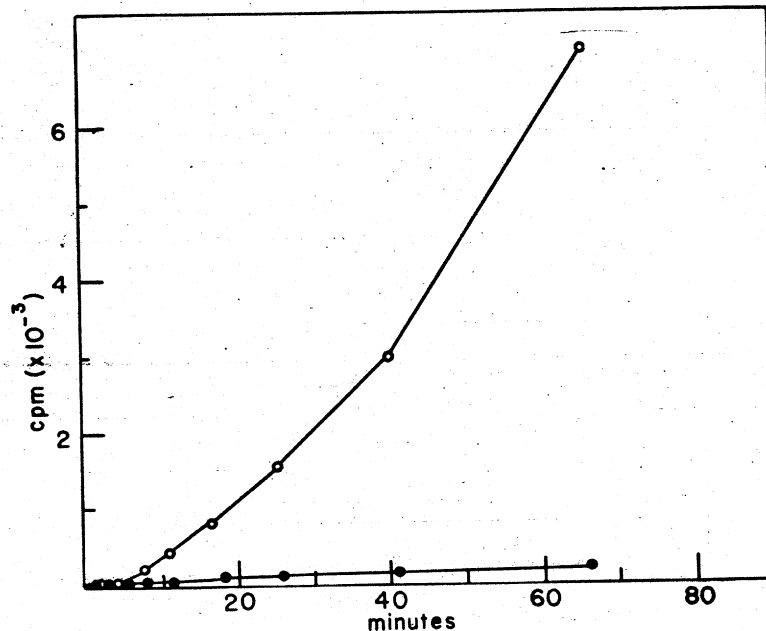
Effect of thionitrates on [^{14}C]uracil incor-

Fig. 2. *Inhibition of* [^{14}C]*uracil incorporation into germinating spores by heat-induced inhibitor. Either the heat-induced inhibitor mixture* (●) *or unheated control* (○) *was added at zero time.*
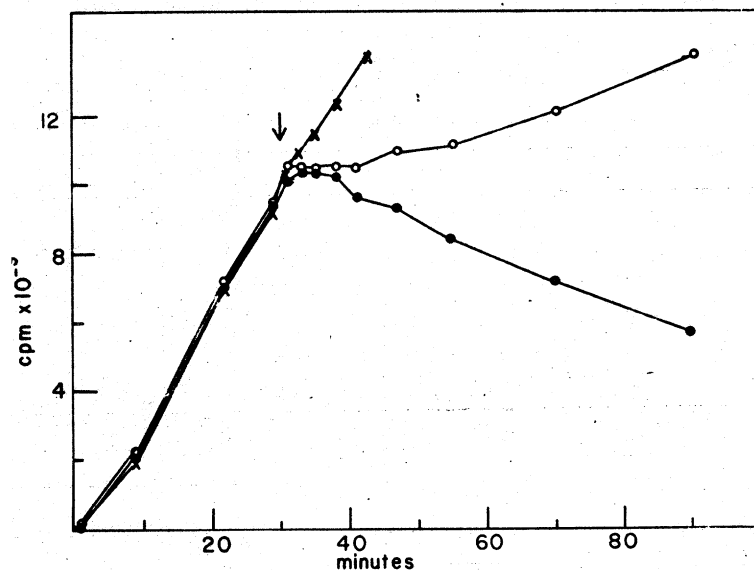
FIG. 3. *Inhibition of* [^{14}C]*uracil incorporation into outgrowing spores by heat-induced inhibitor. Either heated* (●) *or unheated control* (○) *was added 30 min after the onset of germination. A control containing only dialyzable tryptone is also included* (×).
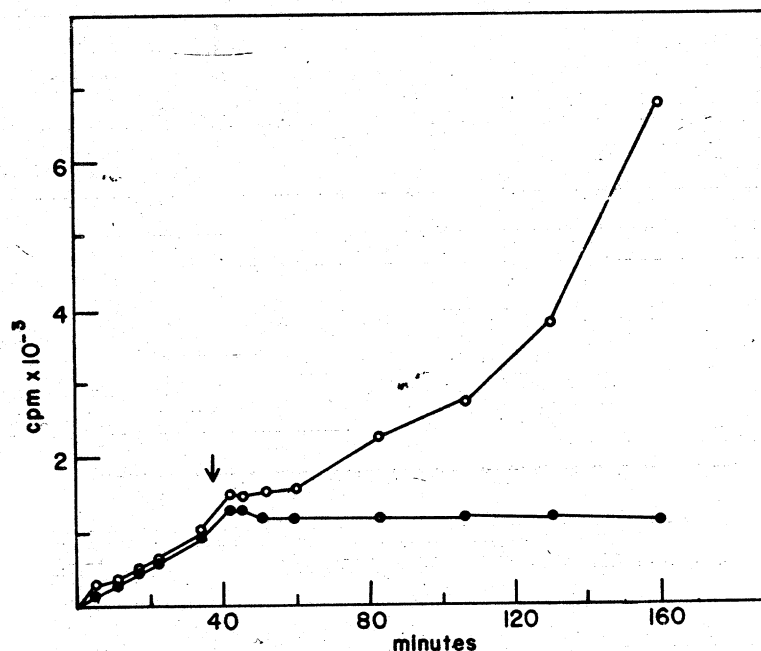

FIG. 4. *Inhibition of* [^{14}C]*uracil incorporation into vegetative cells by heat-induced inhibitor.* [^{14}C]*uracil was added to a vegetative culture in early log phase. After 38 min, either heated* (●) *or unheated control* (○) *was added.*

poration into outgrowing spores. A thionitrite of thioglycolate was prepared as described in Materials and Methods. A control consisted of the same solution, except it was not treated with HCl and was presumed to contain no nitro-

sothiol. Spores were germinated in 10 ml of supplemented CDGS containing [^{14}C]uracil. At the indicated time, 10 ml of the thionitrite was added to one flask and 10 ml of the unacidified control was added to another. Samples were

emoved, and [^{14}C]uracil incorporation was determined as before. Results are shown in Fig. 5. ncorporation of uracil was inhibited by the nitrosothioglycolate. In contrast to the heat-nduced inhibitor, there was no significant drop n trichloroacetic acid-precipitable counts at ater times. This may indicate an inhibition of degradative as well as synthetic systems. When the thionitrite inhibitor was added prior to germination, the morphological events as observed by phase-contrast microscopy were indistinguishable from those shown by the heat-induced inhibitor in Fig. 1. Substitution of the corresponding nitrosothiol of β-mercaptoethanol gave the same results.

Fractionation of precursor to heat-induced inhibitor. The observation that a Perigo-type inhibitor can be formed in media which have previously been sterilized by autoclaving (14) indicates that the precursors are stable to heat, whereas the inhibitor itself is unstable when exposed to air (10). The sensitivity of outgrowing spores to the inhibitor provides a convenient assay which can be used during purification of the precursor. A 100-ml amount of dialyzable tryptone (see above) was applied to a Sephadex G-25 column (5 by 27 cm) which had been equilibrated with water. Fractions of 350 drops each were collected, and the fractions were assayed for their ability to form

inhibitor. To do so, nitrite and thioglycolate were added to 2.5 ml of each fraction (see above) followed by autoclaving at 110 C in 30-ml culture tubes. After cooling the tubes, 1 ml of dialyzable tryptone and 1.5 ml of water were added, followed by heat-shocked spores. The tubes were incubated on a rotating shaker at 25 C. Control cultures reached the vegetative state after about 2 h. Heat-induced inhibitor was assumed to be present when the spores were still inhibited in outgrowth after 4 h. A second observation was carried out after 20 h. Those fractions still inhibited were denoted as containing high levels of inhibitor, whereas those which grew between 4 and 20 h contained low levels. Fractions 13–28 all formed high levels of inhibitor and were pooled. It was noted that the active fractions were the ones in which visible yellow color was most intense.

The pooled material was applied to a diethyl-aminoethyl-cellulose column (Whatman DE-23) which had been equilibrated with 0.01 M potassium phosphate, pH 7.2, at 4 C. The column was washed with 200 ml of buffer, and a linear salt gradient was applied with a total volume of 1,600 ml going from 0 to 0.5 M NaCl in 0.01 phosphate buffer. Precursor activity eluted in the range of 0.25 to 0.35 M NaCl. Salt concentrations were determined by refractive index of the fractions. The active fractions (still yellow)
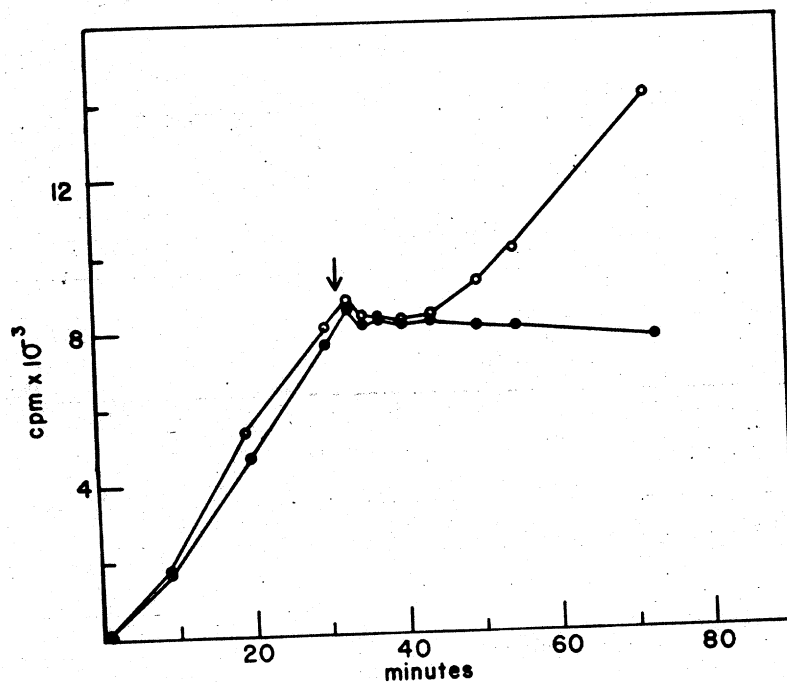

FIG. 5. *Inhibition of [^{14}C]uracil incorporation into outgrowing spores by nitrosothioglycolate. Thirty-one and one-half minutes after the onset of germination, either the nitrosothioglycolate mixture (●) or the "unacidified control" mixture (○) was added.*

were pooled and lyophilized. The residue was dissolved in 15 ml of water and desalted by application to another Sephadex G-25 column (2 by 20 cm) equilibrated with the 0.01 M phosphate buffer. While traversing the column, the yellow color separated into two bands. Figure 6 shows the elution profile of this column. Assays of the fractions showed that precursor activity appeared in fractions 18–23 which coincided with the first yellow peak. The absorbancy at 280 nm (A_{280}) more accurately reflected the presence of precursor than did the protein profile. Since the activity coincided with a yellow color, a 280:400 ratio was also calculated and plotted. This ratio was relatively constant through much of the activity peak, implying a possible relationship between the visible color, the A_{280}, and the precursor activity. Inhibitory activity and a yellow color were also found in the second A_{280} peak. However, inhibition did not require heating (i.e., was not a heat-inducible inhibitor precursor) and coincided with the NaCl peak, which rose to 2 M in fraction 32. The inhibitory activity was attributed to the salt.

The Sephadex G-25 column was standardized by molecules of known molecular weight. Blue dextran, insulin, ribonuclease S peptide, bacitracin, vasopressin, and tritium oxide with molecular weights of about 2 million, 11,500, 2,200, 1,400, 1,000, and 20, respectively, were employed. Sephadex G-25 fractionates, molecules with molecular weights between 1,000 and 5,000 (19). Volumes of 15 ml of each standard were chromatographed, and the elution volume was taken to be the highest point of each peak. Blue dextran and insulin both eluted at fraction 15, which was the exclusion volume. Vasopressin and T_2O both eluted at fraction 33. The log of the molecular weight of the other standards was plotted against the fraction number to give a straight line (19). The activity peak appeared to have a molecular weight of about 2,000. The activity peak was also treated with proteinase K, a broad-spectrum protease (8). Incubation with 50 μg of proteinase K per ml for 1 h at 25 C was ineffective in destroying the ability of the active fractions to form heat-induced inhibitor.

DISCUSSION

The mechanism by which nitrite inhibits food spoilage appears to be quite complex. Many factors, such as medium pH and concentration
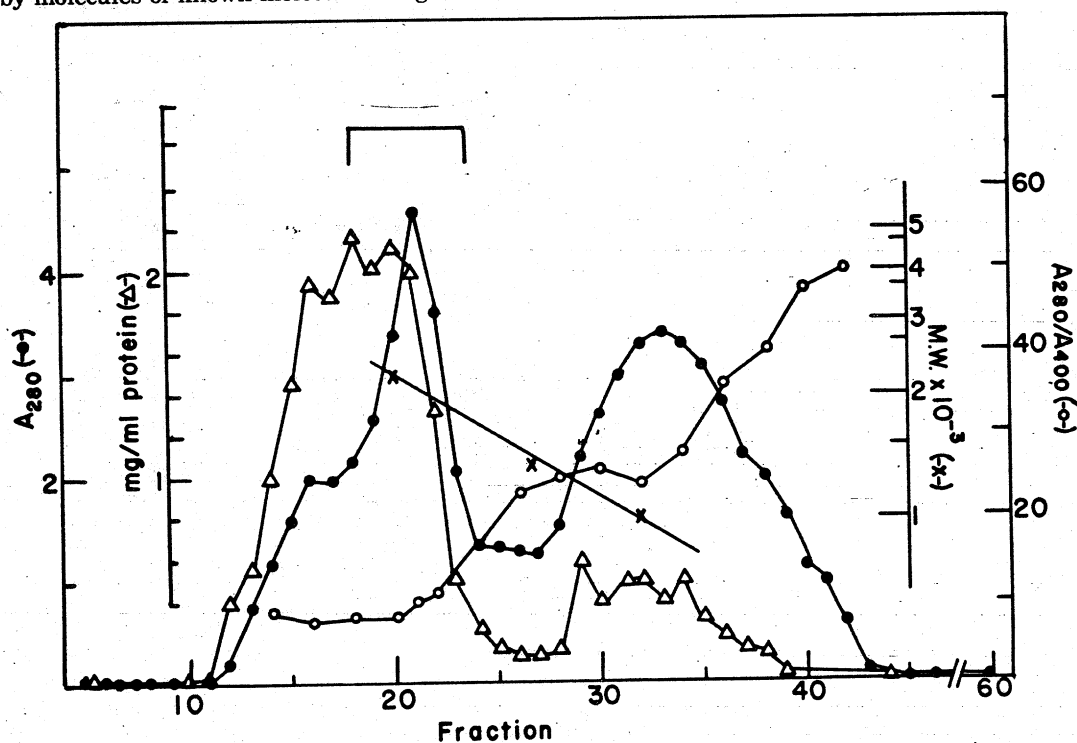

FIG. 6. Profile of Sephadex G-25 column fraction of precursor to heat-induced inhibitor from dialyzable tryptone. The material applied to the Sephadex column was the active peak which was eluted by a NaCl gradient from a diethylaminoethyl-cellulose column. The A_{280} (●), A_{280}-A_{400} ratio (○), and Lowry protein determinations (△) are shown. Also shown from left to right are the molecular weight standards, (×) ribonuclease S peptide (2,200), bacitracin (1,400), and vasopressin (1,000). Precursor activity appeared in fractions 18–23, as indicated by the brackets.

of salts, are important (5, 7, 20). The enhancement of inhibition at low pH has been interpreted to mean that nitrous acid is a reactant intimately involved in the inhibition (6). However, the heat-induced Perigo-type inhibitors are relatively pH independent, suggesting that yet another inhibitor is involved (15). More recently, other nitrite-derived inhibitors such as nitrosothiols (12, 13) and Roussin black salt (1) have been proposed as involved in nitrite action. Media in which inhibitors have been formed contain ether-extractable substances (presumably of low molecular weight) which have spectra which are consistent with these S-nitroso compounds (1).

The numerous chemical species which have thus far been discovered suggest the phenomenon of nitrite inhibition results from the presence of a mixture of inhibitors, all derived from nitrite or its protonated form, nitrous acid. For example, although the formation and efficacy of a Perigo-type inhibitor has been clearly established, its involvement as a food preservative has been questioned (3). However, it may be important as one of several inhibitor components.

The heat-induced inhibitor reported here is of considerable interest. It was formed from the dialyzable fraction of tryptone (Difco), a pancreatic digest of casein. Since Perigo-type inhibitors have been reported as unstable in air (10), we have instead isolated the precursor. It was found to behave as a typical macromolecule in that it adsorbed to a diethylaminoethyl-cellulose ion-exchange column and was eluted by a salt gradient at a discrete salt concentration. Subsequent Sephadex G-25 column chromatography along with molecular weight standards indicated a molecular weight on the order of 2,000. The activity peak coincided with an A_{280} peak which also gave a positive test in the Lowry protein assay. The capacity of the material to subsequently form inhibitor was not appreciably affected by a broad-spectrum protease. Although the molecular weight corresponds to an oligopeptide about 18 residues long, its chemical nature is uncertain. Additional experiments are currently being conducted to establish this. The nondialyzable fraction of the tryptone is also being examined and appears to contain appreciable activity with a higher molecular weight than that obtained from the dialyzable fraction (unpublished data). The purified dialyzable precursor readily formed inhibitor upon heating in the presence of only nitrite and thioglycolate. This means that any required factors such as iron salts must have been present in the purified

fraction. Alternatively, there may be no requirement for such factors.

Although the mode of action of these inhibitors is not yet understood, involvement of sulfhydryl groups as well as a nitroso group is probably important (1, 9, 12, 13). We have observed that both nitrosothiols and the heat-induced inhibitor are very effective in halting incorporation of uracil into ribonucleic acid during stages of outgrowth and vegetative growth. It was further observed that high levels of the heat-induced inhibitor actually prevented germination, and the entire population remained phase-bright, with the exception of a small percentage which partially or completely germinated. Since refractile spores are highly impermeable to chemical agents (21), the inhibitor may react with exterior groups, possibly those (21, 27) which interact with germinating agents. If so, these inhibitors are attractive as probes of the mechanism of germination as well as having potential uses as preservatives.

Our observations, which show that the inhibitors derived from nitrite act at virtually every stage in the life cycle of *Bacillus,* suggest that their mode of action is rather general, and that inhibition may be the result of inactivation of several sensitive metabolic systems or steps. In practice, the presence of several inhibitors, each with a general effect, could be expected to act much more effectively than any alone. Differential inhibitor stabilities and reactivities resulting in a synergistic inhibitory response could help explain the elusive nature of the mode of action of nitrite curing salts used as preservatives. It would be of considerable value to elucidate the molecular mechanism of action of these inhibitors and the differences between them, if any. It might then be possible to design a compound which would combine the desired qualities of stability, effectiveness, and low toxicity.

LITERATURE CITED

1. Ashworth, J., A. Didcock, L. A. Hargreaves, B. Jarvis, and C. L. Walters. 1974. Chemical and microbiological comparisons of inhibitors derived thermally from nitrite with an iron thionitrosyl (Roussin black salt). J. Gen. Microbiol. 84:403–408.
2. Ashworth, B. W., and R. E. Keller. 1967. Ultraviolet determination of tertiary mercaptans as thionitrites. Anal. Chem. 39:373–374.
3. Ashworth, J., and R. Spencer. 1972. The Perigo effect in pork. J. Food Technol. 7:111–124.
4. Barrett, J., D. F. Debenham, and J. Glauser. 1965. The

electronic spectrum and photolysis of S-nitrosotol-uene-α-thiol. Chem. Commun. 12:248–249.

5. Buchanan, R. L., and M. Solberg. 1972. Interaction of sodium nitrite, oxygen and pH on growth of *Staphylococcus aureus*. J. Food Sci. 37:81–85.

6. Castellani, A. G., and C. F. Niven. 1955. Factors affecting the bacteriostatic action of sodium nitrite. Appl. Microbiol. 3:154–159.

7. Church, B. D., H. Halvorson, and H. O. Halvorson. 1954. Studies on spore germination: its independence from alanine racemase activity. J. Bacteriol. 68:393–399.

8. Gross-Bellard, M., P. Oudet, and P. Chambon. 1973. Isolation of high-molecular-weight DNA from mammalian cells. Eur. J. Biochem. 36:32–38.

9. Incze, K., J. Farkas, V. Mihalys, and E. Zukal. 1974. Antibacterial effect of cysteine-nitrosothiol and possible precursors thereof. Appl. Microbiol. 27:202–205.

10. Johnston, M. A., and R. Loynes. 1971. Inhibition of *Clostridium botulinum* by sodium nitrite as affected by bacteriological media and meat suspensions. Can. Inst. Food. Technol. 4:179–184.

11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

12. Mirna, A., and K. Coretti. 1974. Über den Verleib von Nitrite in Fleischwaren. II. Untersuchungen über chemische und bakteriostatische Eigenschaften verschiedener Reaktionsprodukte des Nitrites. Fleischwirtschaft 54:507–509.

13. Mirna, A., and K. Hotmann. 1969. Über den Verleib von Nitrit in Fleischwaren. I. Umsetzung von Nitrit mit Sulfhydryl-Verbindungen. Fleischwirtschaft 49:1361–1364.

14. Nakata, H. M. 1964. Organic nutrients required for growth and sporulation of *Bacillus cereus*. J. Bacteriol. 88:1522–1524.

15. Perigo, J. A., E. Whiting, and T. E. Bashford. 1967. Observations on the inhibition of vegetative cells of *Clostridium sporogenes* by nitrite which has been autoclaved in a laboratory medium discussed in the context of sublethally processed cured meats. J. Food Technol. 2:377–397.

16. Philippe, R. J., and H. Moore. 1961. The infrared spec-

tra of some alkyl thionitrites. Spectrochim. Acta 17:1004–1015.

17. Pivnick, A., H. W. Barnett, H. R. Nordin, and L. J. Rubin. 1969. Factors affecting the safety of canned, cured, shelf stable luncheon meat inoculated with *Clostridium botulinum*. Can. Inst. Food Technol. J. 2:141–148.

18. Rana, R. A., and H. O. Halvorson. 1972. Method for restricting incorporation of radioactivity from [³H]thymidine into deoxyriboncleic acid only during outgrowth of spores of *Bacillus cereus* T. J. Bacteriol. 109:559–605.

19. Reiland, J. 1971. Gel filtration, p. 287–321. *In* B. Jakoby (ed.), Methods in enzymology, vol. 22. Academic Press Inc., New York.

20. Roberts, L. N., and M. Ingram. 1966. The effect of sodium chloride, potassium nitrate and sodium nitrite on the recovery of heated bacterial spores. J. Food Technol. 1:147–163.

21. Roberts, T. A., and A. D. Hitchins. 1969. Resistance of spores, p. 611–670. *In* G. W. Gould, and A. Hurst (ed.). The bacterial spore. Academic Press Inc., New York.

22. Rodenberg, S., W. Steinberg, J. Piper, K. Nickerson, J. Vary, R. Epstein, and H. O. Halvorson. 1968. Relationship between protein and ribonucleic acid synthesis during outgrowth of *Bacillus cereus*. J. Bacteriol. 96:492–500.

23. Saville, B. 1958. A scheme for the colorimetric determination of microgram amounts of thiols. Analyst 83:670–672.

24. Schulz, U., and D. R. McCalla. 1969. Reactions of cysteine with N-methyl-N-nitroso-p-toluenesulfonamide and N-methyl-N′-nitro-N-nitrosoguanidine. Can. J. Chem. 47:2021–2027.

25. Vary, J. C. 1975. Properties of *Bacillus megaterium* temperature-sensitive germination mutants. J. Bacteriol. 121:197–203.

26. Vary, J. C., and H. O. Halvorson. 1965. Kinetics of germination of *Bacillus* spores. J. Bacteriol. 89:1340–1347.

27. Woese, C. R., J. C. Vary, and H. O. Halvorson. 1968. A kinetic model for bacterial spore germination. Proc. Natl. Acad. Sci. U.S.A. 59:869–875.